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Effects of a Composite Endomycorrhizal Inoculum on Olive Cuttings under the Greenhouse Conditions

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Abstract— *This study was carried out in a nursery to evaluate the impact of mycorrhizal fungi on the cutting's root growth, and root colonization of a Moroccan olive variety 'Picholine Marocaine' under greenhouse conditions during 2 years of cultivation. The results revealed that the inoculation with a composite inoculum of arbuscular mycorrhizal fungi (AMF) stimulated an early root formation and high development of vegetative shoots in inoculated cuttings respectively, 35 days (50 days in the control plots) and 40 days (60 days in the control plots) after their culture. The progressive establishment of mycorrhizal symbiosis in the roots of the inoculated plants showed that the root and vegetative masses were respectively 24 g and 19.5 g two years after inoculation. The average height and the leave's number of the inoculated plants relative to the control were respectively s 42/ 12 cm and 145/12.*

The newly formed roots were mycorrhizal and present different structures characteristic of AMF: arbuscules, vesicles, hyphae and spores, whose frequency and intensity reached 90% and 75% two years after cuttings cultivation. The arbuscular and vesicular contents and the number of spores were 67%, 96% and 212 spores/ 100 g of soil respectively. The fourteen species of mycorrhizal fungi isolated from the rhizosphere belong to 4 genera (Glomus, Acaulospora, Gigaspora, and Scutellospora) and three families (Glomaceae, Acaulosporaceae and Gigasporacea).The Glomus genus was the most dominant (65%) followed by the Gigaspora genus (22%). Glomus intraradices, Gigaspora sp.2, Glomus versiformes are the most abundant species, their frequency of occurrence are respectively 30%, 21% and 16%.

Keywords— mycorrhizal fungi, rhizosphere, Scutellospora.

I. MATERIELS AND METHODS

The inoculum consists of the endomycorrhizal species mixture belonging to the genera *Glomus*, *Acaulospora* and *Entrophospora*, originating from the rhizosphere of olive trees growing in different regions of Morocco (Kachkouch *and al*., 2012).

Barley plants were used as a host for the multiplication of mycorrhizal inoculum. Barley seeds were disinfected with Sodium hypochlorite (5%) for two minutes; they were rinsed with tap water and sown in pots containing mycorrhizal soil and mycorrhizal roots fragments of the olive trees. These pots were brought to the greenhouse and watered regularly with distilled water and received 100 mL of a nutritive solution every two weeks. After four weeks of barley cultivation, the roots were recovered and cut into fragments of 1 to 2 cm length.

The olive cuttings are pieces of woody stems, more or less variable in size, taken from trees of 25 years old. 3 grams of mycorrhizal barley root fragments were used as inoculum, applied to the base of each olive cuttings transplanted into a plastic pots containing disinfected soil. Control cuttings are transplanted into soil that does not contain mycorrhizal roots. The pots containing the inoculated and control olive cuttings were subsequently brought in the greenhouse and watered every two to four days with tap water.

Aerial and vegetative part measurements

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Cuttings were harvested 60 days after the experiment start. At each time, the condition of cutting was rated as rooted, callused, or dead. The number of primary and secondary lateral roots on each cutting was counted and removed from the cutting. Fresh weights were obtained from stem and root portions of each cutting using a digital scale. The number of leaves and branches were counted on the vegetative part.

The mycorrhization was assessed on sections of fresh roots of 1 cm after clearing and staining by modified procedure of Philips and Hayman (1970). The mycorrhizal frequency, arbuscular and vesicular contents inside the root bark are measured by assigning an index of mycorrhization from 0 to 5 0: no, 1: trace, 2: less than 10%, 3: 11 to 50%, 4: 51 to 90%, 5: more than 91%.

Mycorrhizal frequency (F %):

F% = 100 × (N0 - n0) / N

With, N: number of observed fragments and n0: number of non-mycorrhizal fragments.

Mycorrhizal intensity (M %):

M% = (95 n5 + 70 n4 + 30 n3 + 5 n2 + n1) / N

With, n = number of fragments assigned with the index 0, 1, 2, 3, 4 or 5.

Arbuscular content (A %):

A% = (100 mA3 + 50 mA2 + 10 mA1) / 100

Where MA3, MA2, MA1 are the percentages (%) respectively assigned to the notes A3, A2, A1, with, MA3 = (95 + 70 n5 n4 A3 A3 + 30 + 5 n2 n3 A3 A3 n1 + A3) / N. The same for A1 and A2.

In this formula, n5A3 represents the number of fragments marked 5 with A3; n4A3 marked the number of fragments 4 with A3; .....

A0: no arbuscules, A1: some arbuscules 10%, A2: moderately abundant arbuscular 50%, A3: very abundant arbuscular: 100%.

Vesicular content (V %):

V% = (100 mV3 + 50 mV2 + 10 mV1) / 100

Where MV3, MV2, MV1 are the percentages (%) respectively assigned notes V3, V2, V1, with, MV3 = (95 + 70 n5 V3 V3 n4 + 30 + 5 n2 n3 V3 V3 n1 + V3) / N. The same for V1 and V2.

In this formula, n5V3 represents the number of fragments marked 5 with V3; n4V3 marked the number of fragments 4 with V3;.....

V0: no vesicles; V1: some vesicles 10% V2: 50% moderately abundant vesicles; V3 abundant vesicles: 100%.

Extraction of spores

The wet sieving method described by Gerdemann and Nicolson (1963) was adopted to extract the spores from the rhizosphere of olive cuttings. A quantity of 100 g of soil was poured into a beaker and then dissolved in 1000 mL of tap water. The resulting solution was left to settle for a few seconds and the suspension was decanted into another beaker, stirred and allowed to stand for 10 to 30 seconds. The suspension was then passed through four superimposed sieves with decreasing mesh size (500, 200, 80 and 50 µm). This operation was repeated twice. The content retained by the sieves of 200, 80 and 50 µm was divided into two tubes and centrifuged for 4 min at 9000 rev / min. The supernatant was discarded and a viscosity gradient created by adding 20 mL of sucrose solution at 40% in each centrifuge tube. The mixture rapidly stirred and the tube was put again in the centrifuge for 1 min at 9000 rpm / min. Unlike the first centrifuging, the supernatant was poured into the sieve of 50 µm, the resulting substrate was rinsed with distilled water to remove sucrose.

The estimation of the number of spores in the soil was made by counting the spores in one mL of supernatant and by the extrapolation of the total volume (100 mL). If no spores were observed, the whole supernatant was reduced to 1 mL and observed again.

II. RESULTS AND DISCUSSION

Inoculated and control olive cuttings developed the first roots in approximately 35 and 60 days respectively after the start of experiment. The first leaves appeared respectively 40 and 60 days in the mycorrhizal and control plants. All inoculated cuttings produce vegetative leaves. On the other hand, only 75% of the control cuttings were able to develop vegetative branches. In the first year after planting of cuttings, the average number of vegetative leaves in inoculated cuttings was 5, with an average length of 30 cm and a number of leaves of 61.7. While, in the non-inoculated cuttings, the mean number of vegetative branches is 2 per cutting with an average height of 3 cm and a number of leaves was 8 (Fig. 1, 2, 3 and 4). In the second year, the average number of vegetative leaves in mycorrhizal cuttings decreased to 10.6 with a length of 42 cm and a number of leaves of 145. The control cuttings had an average of 2 leaves , a length of 12 cm and 12 as the number of leaves (Fig. 2, 3, 4, 5).

The mean vegetative biomass was in the order of 19.5 g after two years in inoculated cuttings compared to 0,9g in controls. The development of vegetative biomass is correlated with increasing of the root system. Indeed, rooting was greater on cuttings inoculated with endomycorrhizal inoculum than in non inoculated plantss with a large root mass on average of 24 g per cutting (Figure 6).

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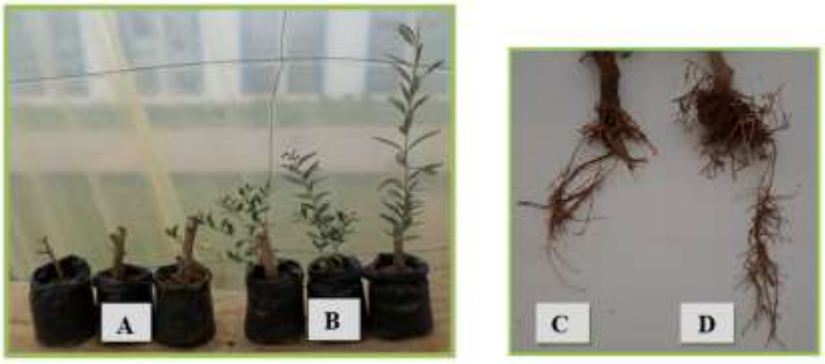

Fig. 1 : Aerial part of control (A);inoculated olive cuttings one year after inoculation (B); Root part of control (C); Inoculated (D) olive cuttings 1 year after inoculation.

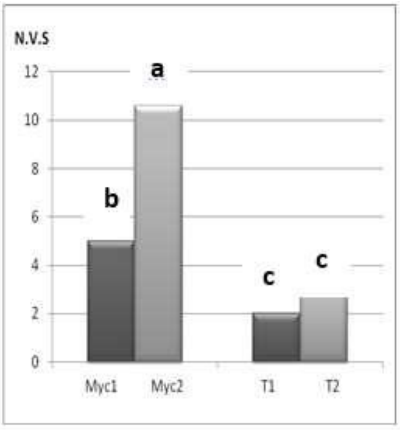

Fig.2: Average number of vegatative buds in olive mycorrhizal cuttings 1year (Myc 1) and 2years (Myc 2) after cultivation and in control cuttings after 1 (T1) and 2 years (T2).

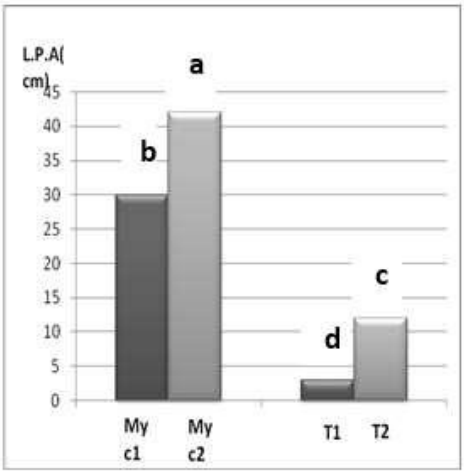

Fig.3: Average lenght of vegatative buds in olive mycorrhizal cuttings after 1year (Myc 1) and 2years (Myc 2) after cultivation and in control cuttings after 1 (T1) and 2 years (T2).

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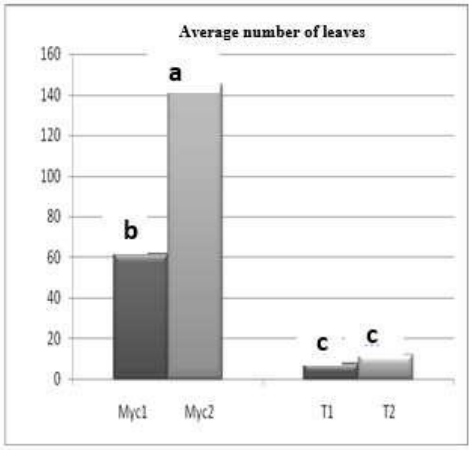

Fig. 4: Average number of leaves of mycorrhizal olive cutting after 1year (Myc 1) and 2years (Myc 2) after cultivation and in control cuttings after 1 (T1) and 2 years (T2).

Fig. 5: Aerial and root parts of the of the control cutting 1 year after cultivation (A), (B); Arial and root parts of the inoculated Picholine olive cutting 1 year after cultivation; (C) Aerial and root parts of the control Picholine olive cutting 2 years after cultivation; (D) Aerial and root parts of the inoculated Picholine olive cutting 2 years after cultivation.

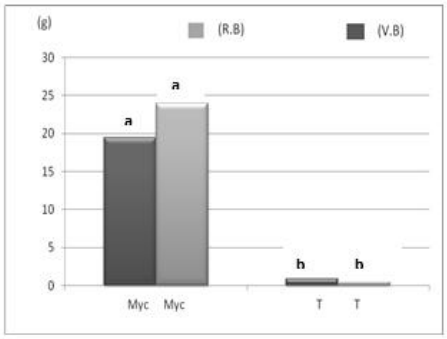

Fig. 6: Vegetative fresh biomass (V.B.) and root fresh biomass (R.B.) in mycorrhizal olive cuttings (Myc) or not (T) 2 years after cultivation. Inoculation.

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Table.1: Effect of mycorrhization on aerial and root growth of olive cuttings.

Olive Cuttings / Parameters	Mycorhizal cuttings	Control cuttings
Leave's number	145	12
Vegetative buds number	10.6	2
Average length of vegetative part (cm)	42	12
Root fresh biomasse (g)	24	0.4
Vegetative fresh biomasse (g)	19.5	0.9

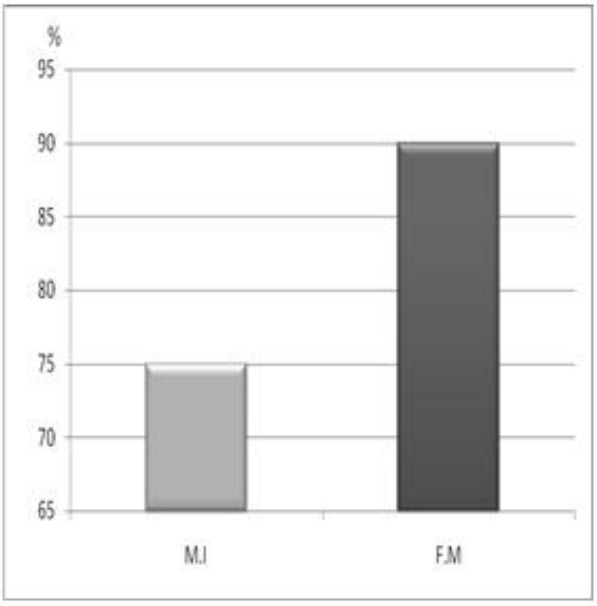

Fig.7: Mycorrizal Intensity (MI) and frequency (FM) in roots of olive cuttings after 2 years ofinoculation.

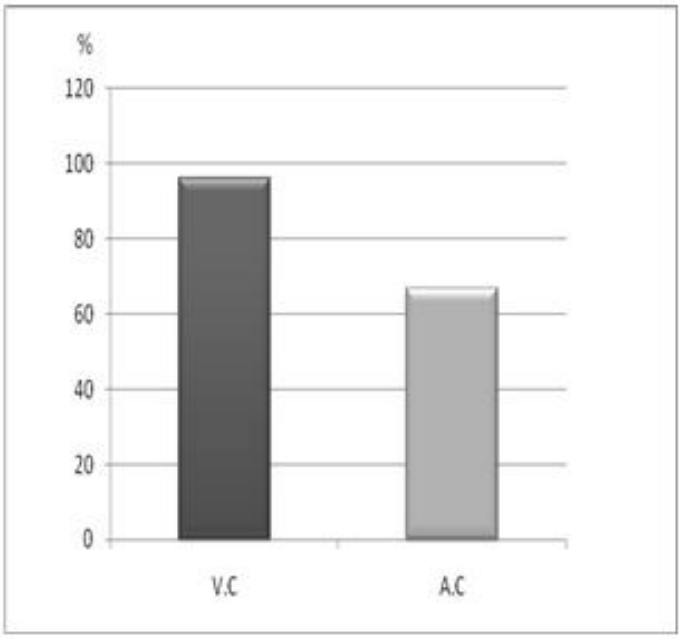

Fig. 8: Vesicular and arbuscular contents in roots of olive cuttings after 2 years of inoculation.

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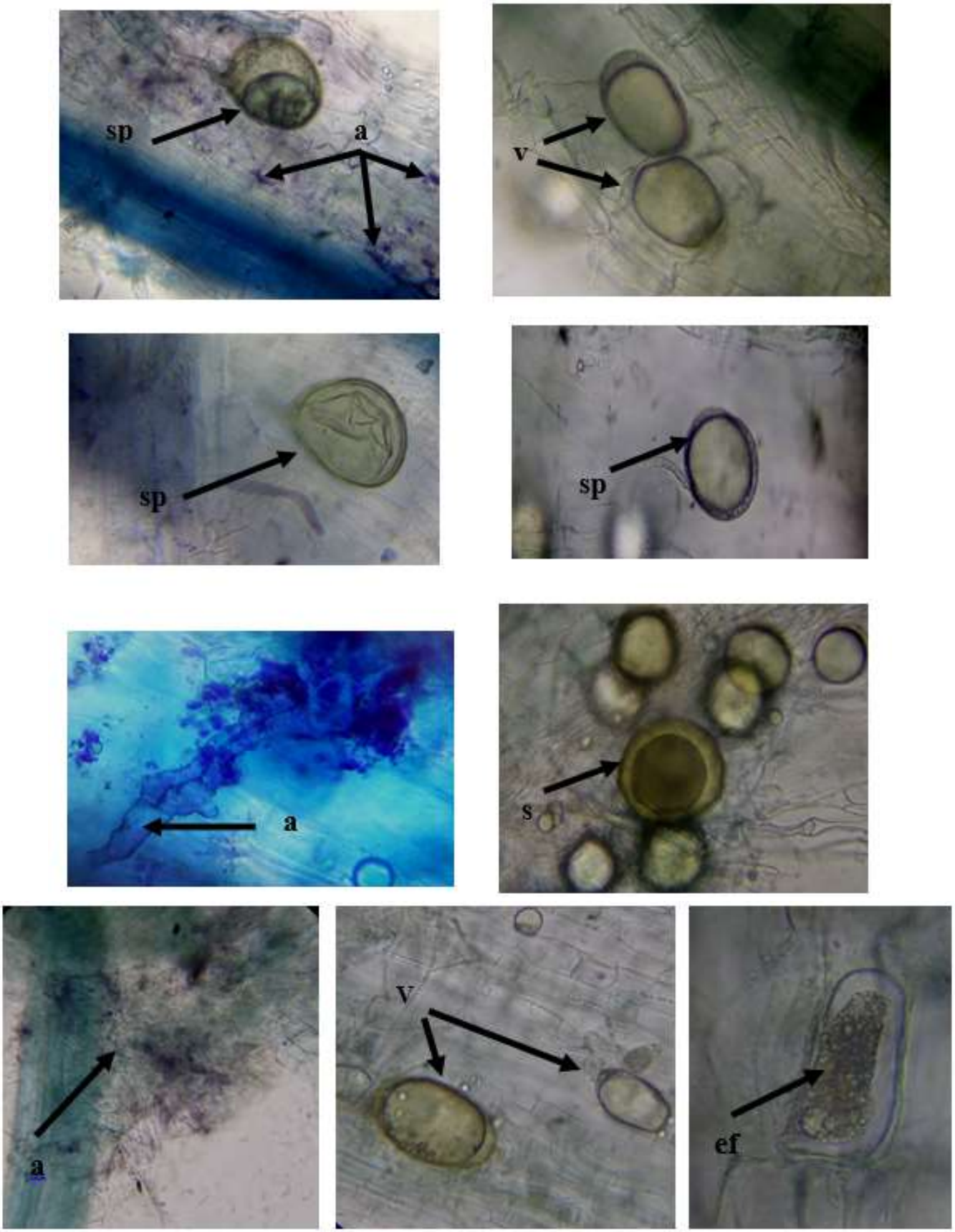

Fig.9: Endomycorrhizal structures formed in the association of mycorrhizae/olive tree cuttings. sp: spores ;v: vesicles ; ef: endophytes ; a: arbuscules. G × 400.

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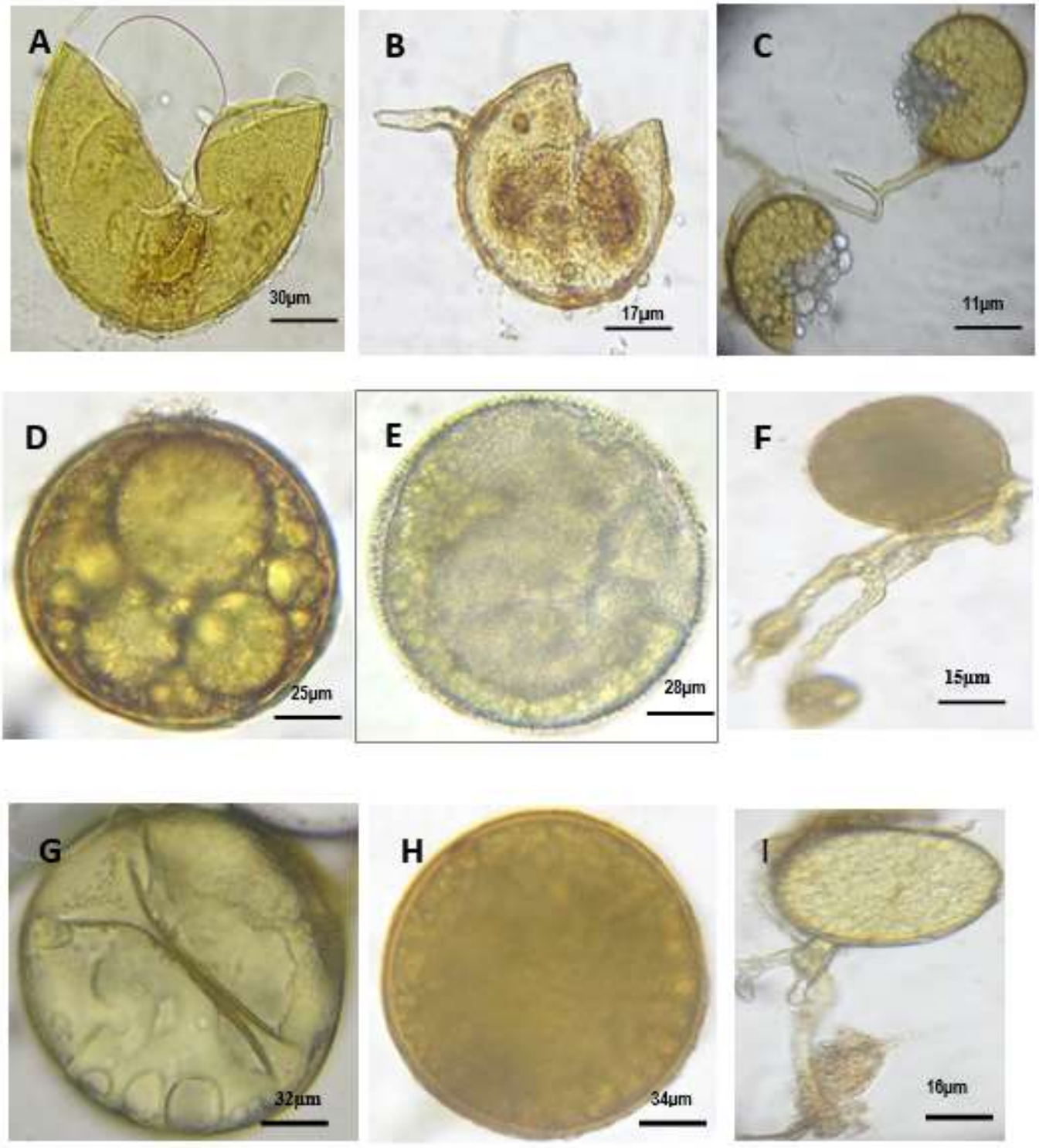

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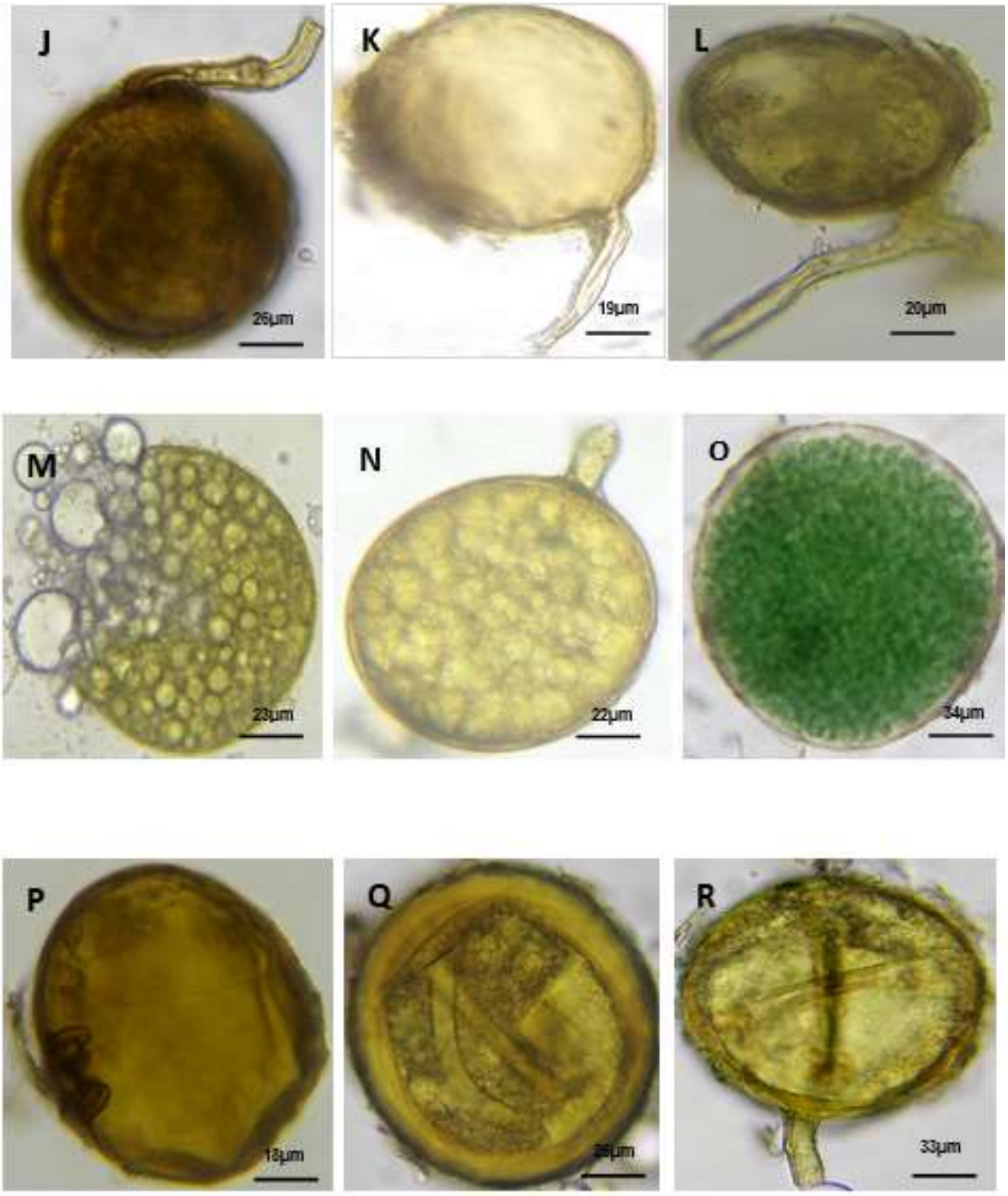

Fig. 10 : Endomycorrhizal spores isolated from the rhizosphere of mycorrhizal olive cuttings (×400). A: Acaulospora scrobiculata ; B :Glomus macrocarpum; (C,L,M ,N) : Glomus intraradices; D : Glomus versiforme; E: gigaspora sp.1, F,H:Glomus clarum, G: Scutellospora fulgida; I: Glomus aureum; J: Glomus aurantium; K: Glomus geosporum; O :Gigaspora sp.2; P: Scutellospora heterogama; Q: Glomus corymbiforme; R: Glomus microcarpum.

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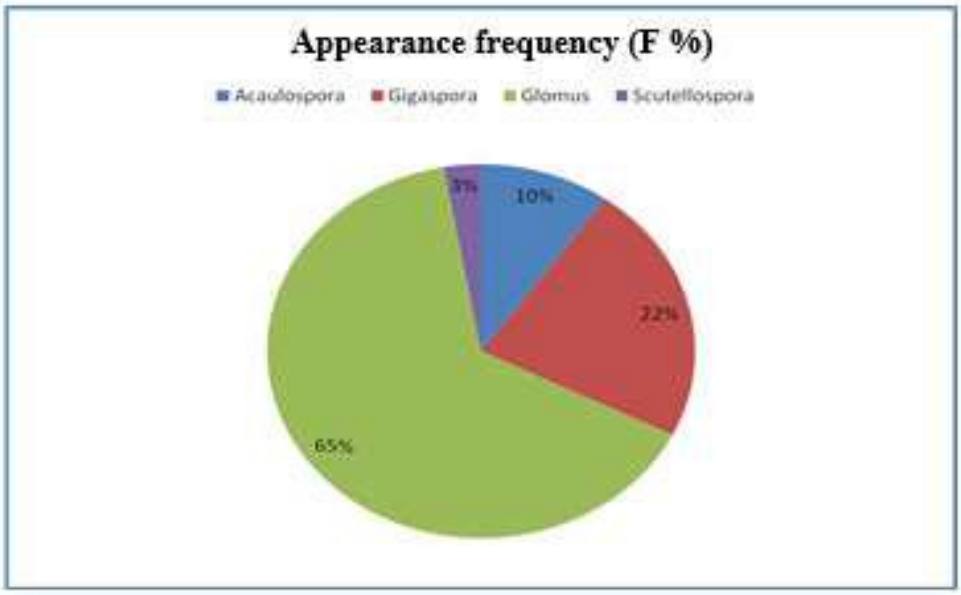

Fig. 11: Appearance frequency of all genera associated with the rhizosphere of olive cuttings inoculated with a composite endomycorrhizal inoculum.

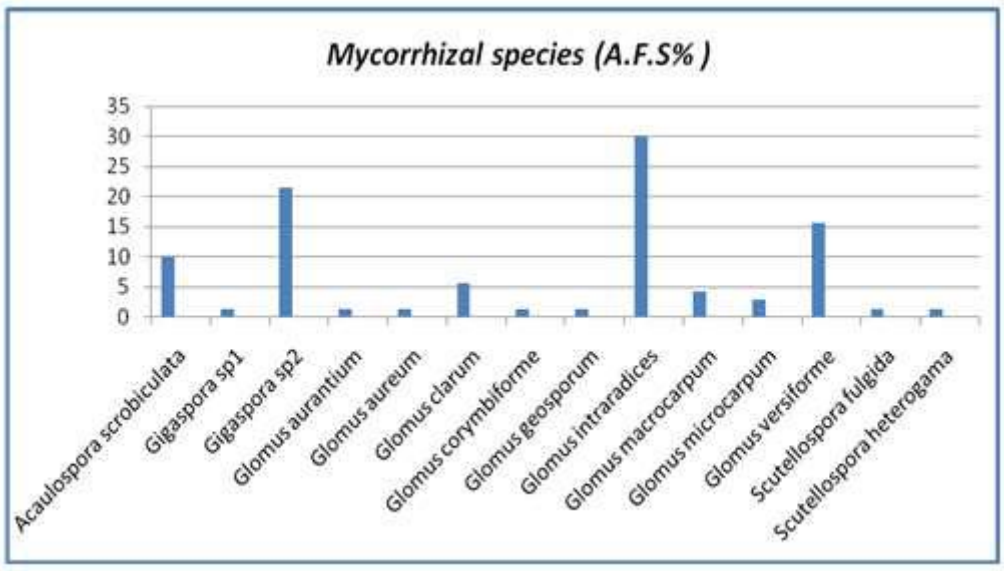

Fig. 12: Isolation frequency of mycorrhizal species in the rhizosphere of olive cuttings inoculated with mycorrhizae after 20 months of culture.

The root system of the mycorrhizal cuttings was more developed, with adventitious and lateral roots of the 1st, 2nd and 3rd order (Figure 5). In contrast, control olive cuttings showed a lower development in the root system of 0.4 g per cutting (Figure 6).

The data in table 1 demonstrated the positive effect of the endomycorrhizal species on different growth parameters of the aerial parts of inoculated olive cuttings that showed greater development than controls.

The evaluation of mycorrhizal root colonization of the olive cuttings showed an important intensity and frequency of mycorrhization reaching respectively 75% and 90% (Figure 7).

Also the arbuscular and vesicular content of mycorrhizal cuttings were respectively 67% and 96% (Figure 8). Microscopic observation of root fragments showed that the inoculated plants were successfully mycorrhizal. These observations also revealed the presence of endophytes, hyphae, arbuscules and vesicles (Figure 9). The revealed spore's density in the olive's cutting rhizosphere was 212 spores / 100 g of soil.

The isolation of mycorrhizal fungi from the rhizosphere soil has allowed to note the presence of 14 species of

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endomycorrhizal fungi; *Acaulospora scrobiculata, Glomus macrocarpum, Glomus intraradices, Glomus versiforme, Gigaspora* sp. 1, *Glomus clarum, Scutellospora fulgida, Glomus aureum, Glomus aurantium, Glomus geosporum, Gigaspora* sp. 2, *Scutellospora heterogama, Glomus corymbiforme, Glomus microcarpum* (Figure 10) belonging to 4 genera: *Glomus, Acaulospora*, *Gigaspora*; and *Scutellospora* (Fig 11, 12) with a dominance of *Glomus* which presents 65% of the isolated species.

Thus, the study of the effects of composite endomycorrhizal inoculum on the rooting of woody cuttings of the olive plants has proved its advantageous impact on growth of roots, aerial parts, shoots and leaves in the early stage of cultivation. The presence of endomycorrhizal fungi with the cuttings probably stimulated their early root production and the progressive development over time, of the young formed roots explains the important effect observed on the growth of the roots and vegetative buds, expressed by the root and aerial fresh weight, the vegetative part length, the number of vegetative branches and the number of leaves.

Some studies have shown that mycorrhizae, by the development of the mycelium, modify the general architecture of the roots (Smith and Read, 1997; Berta *et al*., 1995). Indeed, the improvement of ramification of root system by mycorrhizal fungi was proved on other hosts, *Vitis vinifera* (Schellenbaum *et al.,* 1991), *Populus* sp. (Hooker *et al.,* 1992 ; Nounsi *et al.,2015*), *Platanus acerifolia* (Tisserant *et al.,* 1992), *Prunus ceracifera* (Fortuna *et al.,* 1998), *Coffea arabica* (Al-Areqi *et al.,*2014), *Olea europaea* (Chliyeh *et al.,* 2014) and *Ceratonia siliqua* (Talbi *et al.,* 2015).

Moreover, using the endomycorrhizal fungi in the rooting substrate increase the success rate of cuttings. The recorded percentage was 100% which remained stable after 24 months of cultivation, but in control cuttings, it was lower, and varied respectively from 45% to 75% after 12 and 24 months after plantation.

Concerning, non rooted control cuttings and those having not enough developed roots, they died during 6 weeks and the first year of cuttings respectively. Such findings remains inexplicable at the moment, but there is a trend to get sure that cuttings mortality can only be related to inoculations with endomycorrhizal fungi. Indeed, the noted root and vegetative biomass for some non-inoculated cuttings after their survival and rooting was lower than that observed amongst mycorrhizal cuttings. The presence of endomycorrhizal fungi in the rooting substrate therefore influences the quality of rooting. However, there is conflicting information on the influence of mycorrhizal fungi on rooting of cuttings. Nelson (1987), for example, he noted that colonization of the roots of certain ornamental woody plants species by the mycorrhizal fungi does not mean that the symbiosis is sufficiently functional for the plant to derive all its benefits. It has been noted that sometimes the colonization of the roots is slow, take up 3 or 5 months, and depends on the plant species Nelson (1987). Tréparnier (1998) reported that when propagules of endomycorrhizal fungi are distributed throughout the growing substrate, cuttings take longer time to be colonized, as roots must first pass close to a spore. According to this author, the location of the propagules of fungi in direct contact with the base of the stem as soon to rooting could reduce the duration of infections and thus, the effects on growth would be more perceptible. In the same context, Douds *and al*. (1995) obtained significantly increased survival and a better percentage of rooting for *Sciadopitys verticillata* cuttings in a substrate containing *Glomus intraradices* propagules. In agreement with our results, vesicular and arbuscular mycorrhizal fungi (VAMF) colonization has shown to increase the survival and growth of rose explants (Wilson *and al*., 1997) and strawberry transplants (Chavez and Cerrato, 1990). Verkade *and al*.,(1988) found that inoculation of *Cornus sericea* cuttings with *Glomus fasciculatum* substantially increased plant growth during later stage of development. In soil substrates lacking indigenous mycorrhizal fungi, mycorrhizal inoculation has been found to increase crop uniformity, reduce transplant mortality and increase productivity of geranium (Biermann and Linderman**,** 1983), onion (Vosatka, 1995), *Cyclamen persicum*, *Euphorbia pulcherrima* (Vosatka *et al*., 1999). In other experiment, the study of the effect of mycorrhizal fungi on the rooting of miniature rose cuttings demonstrate that the root colonization resulting from inoculation could result in a higher quality cutting that is able to withstand the stress of transplanting and increase growth during later stage of plant development. Indeed, the positive effect on plant growth was attributed to improved absorption of water and nutrients provided by the extended fungal hyphae and the increased root length and density (Bethlenfalvay *et al.*, 1988; Faber *et al.*, 1991; Fouad *et al.*, 2012, 2013; Essahibi *et al.,* 2013).

In conclusion**,** the used endomycorrhizal inoculum showed a positive effect both on early rooting of woody cuttings and on the formation of vegetative branches. The advantage of mycorrhization increased during the first year and the second year of cultivation giving the improvement of root structure and enhanced fresh root and vegetative part weight of olive cuttings over time. Additionally, our experiments prove the efficiency of endomycorrizal inoculum to support the survival of cuttings and its rooting. Moreover, in function of time, the mycorrhization rate increases and became more effective.

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